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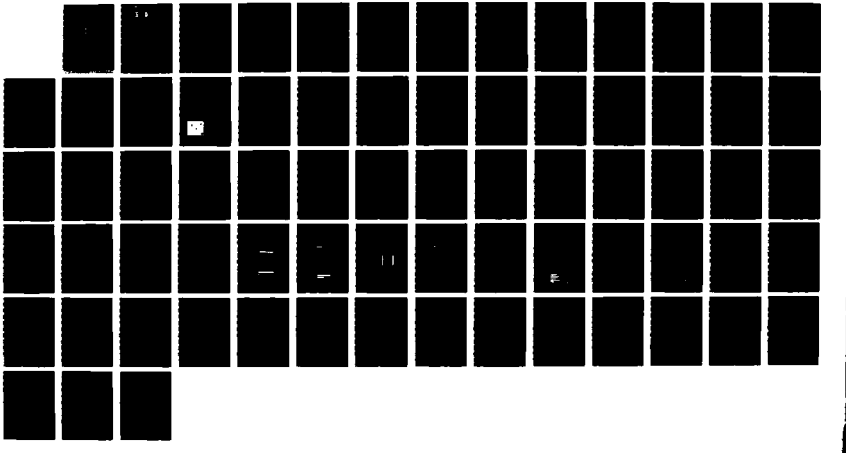

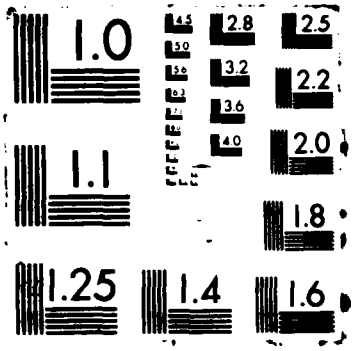
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The purpose of this contract was to analyze the immune responses to dengue infections. During the past few years we have focused on three immune responses; (i) lysis of dengue virus-infected cells by peripheral blood mononuclear cells (PBMC) of non-immune donors; (ii) antibody-dependent complement-mediated lysis of dengue virus-infected cells; (iii) interferon induction from PBMC by dengue virus.

Peripheral blood mononuclear cells (PBMC) from humans without antibodies to dengue 2 virus lysed dengue 2 virus-infected Raji cells to a significantly greater degree than uninfected Raji cells. Addition of mouse anti-dengue antibody increased the lysis of dengue-infected Raji cells by PBMC. These results indicate that both PBMC-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) can cause significant lysis of dengue infected Raji cells. The lysis of infected Raji cells in the ADCC assay correlated with the dilution of dengue-specific antibody which was added, indicating the dengue virus specificity of the lysis of dengue virus-infected Raji cells. Alpha interferon

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(IFNα) was detected in the culture supernatant of PBMC and dengue-infected Raji cells. However, enhanced lysis of dengue-infected Raji cells by PBMC may not be due to the IFN produced, because neutralization of all IFN activity with anti-IFNα antibody did not decrease the lysis of dengue-infected cells.

The effector cells responsible for lysis of dengue virus-infected Raji cells in the NK and ADCC assays were analyzed. Non-adherent peripheral blood lymphocytes (PBL) caused more lysis than did adherent cells. The PBL active in lysing dengue virus-infected Raji cells were characterized using monoclonal antibodies and were compared to lymphocytes that lysed K562. Leu11+ cells lysed dengue virus-infected cells and K562 cells. Leu11- cells lysed dengue virus-infected cells, but not K562 cells. In the Leu11+ fraction, Leu11+ Leu7- cells were more active than Leu11+Leu7+ cells in lysing dengue virus-infected cells. T3+ cells also lysed dengue virus-infected cells, but they did not lyse K562 cells. T3-cells lysed both target cells. These results, along with the observation that Leu11+ cells and T3+ cells are different subsets of PBL, indicate that the PBL that are active in lysing dengue virus-infected cells are heterogeneous and are contained in Leu11+ and T3+ subsets. Leu11+ cells are more active than T3+ cells. Leu11+ cells are active in lysing dengue virus-infected cells by antibody-dependent cell-mediated cytotoxicity, whereas T3+ cells are not active.

Dengue virus-infected cells were lysed by antibody and complement. Although constant lysis of dengue virus-infected cells were observed, the percent specific lysis was about ten percent at every experiment.

We also analyzed the interaction between the peripheral blood lymphocytes (PBL) or non-immune donors and dengue virus-infected cells, which results in interferon (IFN) production. Autologous monocytes or the Epstein-Barr virus transformed B lymphoblastoid cell line (Raji cells) infected with dengue virus were used as IFN inducer cells. PBL produced IFN when cultured with dengue virus-infected cells. Dengue virus-infected cells treated with glutaraldehyde or paraformaldehyde, which produced no infectious dengue virus, also induced IFN. These results indicate that PBL produced IFN in response on dengue virus-infected cells and that the production of IFN by PBL is due to stimulation of PBL by dengue virus-infected cells. The ability of dengue-infected cells to induce IFN correlated with the appearance of dengue antigens in infected cells. Characterization of IFN-producing PBL with monoclonal antibodies demonstrated that the IFN-producing cells were heterogeneous. The predominant IFN producing cells were contained in HLA-DR+, M1+ and T3- subsets. The Leu11+ subset and Leu12+ subsets also contained some IFN- producing cells. The IFNs that were produced by the PBL exposed to dengue virus-infected cells were analyzed by radioimmunoassay employing monoclonal antibodies to detect specifically IFNα or IFNγ. IFNα was the predominant IFN produced. In addition, dengue-monocytes induced low titers of IFNγ in some experiments, and when dengue virus-infected Raji cells as inducer cells, IFNγ was detected in all the experiments. To determine whether the levels of IFN which were detected could prevent dengue virus infection, monocytes were treated with 400IU/ml of IFNα before infection. Treatment of monocytes with IFN decreased the yield of infectious virus more than 99% and the percentage of dengue-antigen positive cells by 98%. These results suggest that IFNs produced by PBL in response to dengue virus-infected cells may have an important role in controlling dengue infection and in the pathogenesis of dengue hemorrhagic fever and shock syndrome.

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HUMAN IMMUNE RESPONSES TO DENGUE VIRUSES

FINAL REPORT

FRANCIS A. ENNIS

AUGUST 1, 1986

Supported by

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SUMMARY

The purpose of this contract was to analyze the immune responses to dengue infections. During the past few years we have focused on three immune responses; (i) lysis of dengue virus-infected cells by peripheral blood mononuclear cells (PBMC) of non-immune donors; (ii) antibody-dependent complement-mediated lysis of dengue virus-infected cells; and (iii) interferon induction from PBMC by dengue virus.

Peripheral blood mononuclear cells (PBMC) from humans without antibodies to dengue 2 virus lysed dengue 2 virus-infected Raji cells to a significantly greater degree than uninfected Raji cells. Addition of mouse anti-dengue antibody increased the lysis of dengue-infected Raji cells by PBMC. These results indicate that both PBMC-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) can cause significant lysis of dengue-infected Raji cells. The lysis of infected Raji cells in the ADCC assay correlated with the dilution of dengue-specific antibody which was added, indicating the dengue virus specificity of the lysis of dengue virus-infected Raji cells. Alpha interferon (IFNα) was detected in the culture supernatant of PBMC and dengue-infected Raji cells. However, enhanced lysis of dengue-infected Raji cells by PBMC may not be due to the IFN produced, because neutralization of all IFN activity with anti-IFNα antibody did not decrease the lysis of dengue-infected cells.

The effector cells responsible for lysis of dengue virus-infected Raji cells in the NK and ADCC assays were analyzed. Non-adherent peripheral blood lymphocytes (PBL) caused more lysis than did adherent cells. The PBL active in lysing dengue virus-infected Raji cells were characterized using monoclonal antibodies and were compared to lymphocytes that lysed K562 cells. Leu11$^+$ cells lysed dengue virus-infected cells and K562 cells. Leu11$^-$ cells lysed

dengue virus-infected cells, but not K562 cells. In the Leu11$^+$ fraction, Leu11$^+$Leu7$^-$ cells were more active than Leu11$^+$Leu7$^+$ cells in lysing dengue virus-infected cells. T3$^+$ cells also lysed dengue virus-infected cells, but they did not lyse K562 cells. T3$^-$cells lysed both target cells. These results, along with the observation that Leu11$^+$ cells and T3$^+$ cells are different subsets of PBL, indicate that the PBL that are active in lysing dengue virus-infected cells are heterogeneous and are contained in Leu11$^+$ and T3$^+$ susets. Leu11$^+$ cells are more active than T3$^+$ cells. Leu11$^+$ cells are active in lysing dengue virus-infected cells by antibody-dependent cell-mediated cytotoxicity, whereas T3$^+$ cells are not active.

Dengue virus-infected cells were lysed by anti-dengue antibody and complement. Although constant lysis of dengue virus-infected cells were observed, the percent specific lysis was about ten percent at every experiment.

We also analyzed the interaction between the peripheral blood lymphocytes (PBL) of non-immune donors and dengue virus-infected cells, which results in interferon (IFN) production. Autologous monocytes or the Epstein-Barr virus transformed B lymphoblastoid cell line (Raji cells) infected with dengue virus were used as IFN inducer cells. PBL produced IFN when cultured with dengue virus-infected cells. Dengue virus-infected cells treated with glutaraldehyde or paraformaldehyde, which produced no infectious dengue virus, also induced IFN. These results indicate that PBL produced IFN in response on dengue virus-infected cells and that the production of IFN by PBL is due to stimulation of PBL by dengue virus-infected cells. The ability of dengue-infected cells to induce IFN correlated with the appearance of dengue antigens in infected cells. Characterization of IFN-producing PBL with monoclonal antibodies demonstrated that the IFN-producing cells were heterogeneous. The predominant IFN producing cells were contained in HLA-DR+, M1+ and T3-

subsets. The Leu11+ subset and Leu12+ subsets also contained some IFN-producing cells. The IFNs that were produced by the PBL exposed to dengue virus-infected cells were analyzed by radioimmunoassay employing monoclonal antibodies to detect specifically IFNα or IFNγ. IFNα was the predominant IFN produced. In addition, dengue-infected monocytes induced low titers of IFNγ in some experiments, and when dengue virus-infected Raji cells as inducer cells, IFNγ was detected in all the experiments. To determine whether the levels of IFN which were detected could prevent dengue virus infection, monocytes were treated with 400IU/ml of IFNα before infection. Treatment of monocytes with IFN decreased the yield of infectious virus more than 99% and the percentage of dengue-antigen positive cells by 98%. These results suggest that IFNs produced by PBL in response to dengue virus-infected cells may have an important role in controlling dengue infection and in the pathogenesis of dengue hemorrhagic fever and shock syndrome.

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I. Introduction

Dengue virus infection is a major world wide cause of morbidity, and dengue hemorrhagic fever (DHF) dengue shock syndrome (DSS) are severe and, at times fatal complications of dengue infections (1). These complications are more commonly observed in individuals undergoing a secondary dengue infection with a virus of another dengue sterotype than that which they experienced in their primary infection (2). These observations indicate that the immune response of the host may play an important role in the complications of dengue infection.

In primary dengue infection, however, most of the patients recover from dengue fever without severe complications. The immune mechanisms which are effective in the recovery from primary dengue virus infection are not known. These two observations suggest that the role of immune response to dengue virus in vivo may be very complex: (i) Some immune responses are effective and most patients recover from dengue infection, (ii) Other immune responses may induce severe complications of dengue infection. In order to understand the possible roles of immune responses in the recovery from or in the pathogenesis of dengue infection, we need to elucidate immune responses, in dengue virus infection.

During the past few years we have focused on three immune responses. These are (i) PBMC-mediated lysis of dengue virus-infected cells, (ii) complement-mediated lysis of dengue-infected cells, (iii) interferon induction by dengue virus.

We have found that (i) dengue virus-infected cells are lysed by PBL to a greater degree than uninfected cells. Both natural killing (NK) and antibody-dependent cell-mediated cytotoxicity (ADCC) are active in killing dengue virus-infected cells. The active effector cells are heterogeneous and the

most predominant effector cells are characterized as Leu11+ cells. (ii) Dengue virus-infected cells are lysed by antibody-dependent, complement-mediated mechanism, however, the percentage of lysis by this mechanism is low. (iii) Dengue virus induces IFN from PBMC by two mechanisms. Dengue virus-infected monocytes produce IFN. These dengue-infected monocytes induce IFN from autologous non-immune PBL. The levels of IFN which were induced from PBMC by dengue virus can inhibit the further infection of dengue virus to human monocytes.

In the next few years we will analyze the HLA restricted cytotoxic T lymphocyte (CTL) response to dengue virus infections to determine the dengue antigenic specificity of these CTL responses and whether the CTL response correlates to the humoral antibody responses, protection against dengue infection or immunopathology, as seen in DHF and DSS. Studies will also be performed to analyze the humoral antibody responses to dengue virus infection. Dengue specific complement-dependent lytic antibodies (CDLA) have been detected using convalescent sera from dengue patients. The specificity of this antibody will be analyzed, and the relationship between this antibody, and neutralizing or infection-enhancing antibodies will be assessed. These cellular and antibody assays will be used to analyze the immune responses to natural primary and secondary dengue infections, and to immunization with experimental vaccines. The results obtained from these studies should help to decrease the potential adverse effects from vaccines to prevent dengue infections, and to develop effective vaccines against dengue.

II. Lymphocytes-mediated lysis of dengue virus-infected cells

IIA. Lysis of dengue virus-infected cells by PBMC and by antibody-dependent cell-mediated cytotoxicity (ADCC)

(1) Preparation of Raji cells persistently infected with dengue 2 virus as infected target cells

Raji cells were infected with dengue 2 virus at a MOI of 0.05 PFU per cell at 37° for 2 hours. Infected cells were washed twice with RPMI, resuspended at the concentration of 2 x 10^5 cells/mL in RPMI/10% FCS and cultured at 37°C in 5% CO_2. The cells were suspended in fresh medium (RPMI/10% FCS) at the concentration of 2 x 10^5 cells/mL every three days. Seven days after infection, 40 percent of the cells were positive and by the ninth day 90 percent were positive for dengue membrane and cytoplasmic antigens. These cells were split every three days at a ratio of 1:10 and have remained infected over one year, with more than 90 percent expressing membrane and cytoplasmic dengue antigens (Figure 1). The cells in this persistently infected culture line are more than 95 percent viable as determined by dye exclusion testing with trypan blue. The dengue virus titer of supernatant culture fluids is 3 x 10^3 PFU/ml. These persistently infected Raji cells were used as target cells in the following studies.

(2) Lysis of dengue 2-infected Raji cells by PBMC with or without anti-dengue 2 antibody

Dengue 2-infected Raji cells and uninfected Raji cells were used as target cells with normal human PBMC as effector cells. These PBMC were obtained from healthy donors who do not have antibodies to dengue virus. Dengue-infected

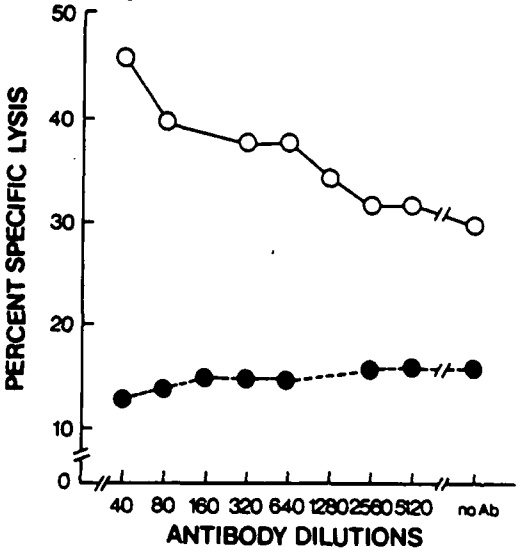
Raji cells were lysed by PBMC without antibody to a greater degree than uninfected Raji cells ($p<0.01$) (Table 1).

There is statistically significant correlation in the specific lysis by PBMC between K562 cells and dengue-infected cells. However, there is a relatively high heterogeneity as to the lytic activity of PBMC against dengue-infected cells, and some effector cells which lyse K562 to an average level lyse dengue-infected cells to a low level.

Addition of anti-dengue 2 antibody significantly increased the lysis by PBMC of dengue 2-infected cells ($p<0.0025$), but not that of uninfected Raji cells (Table 1). There was a dose-response relationship between the added antibody and the specific ^{51}Cr release by ADCC, when hyperimmune ascitic fluid was used. Normal ascitic fluid did not increase the level of lysis of dengue virus-infected cells or uninfected cells (Figure 2). These results indicate that PBMC lyse dengue-infected Raji cells to a greater degree than uninfected Raji cells and that the augmentation of PBMC-mediated killing by anti-dengue antibody was detected on dengue-infected target cells but not on the uninfected target cells.

Figure 1. Fluorescence staining of dengue virus-infected Raji cells.

Figure 2. Dose-response relation between ADCC-lysis and the dose of antibody.



○:Dengue virus-infected Raji cells were used as target cells.

●:Uninfected Raji cells were used as target cells.

Table 1

Lysis of dengue 2-infected Raji cells by PBMC and antibody

Effector	% Specific ^{51}Cr release*				
	K562	Infected Raji***		Uninfected Raji***	
(E/T = 50)		-Ab	+Ab**	-Ab	+Ab
1	14.6	7.7	30.5	5.3	1.7
2	37.6	8.6	29.4	7.8	7.3
3	47.6	18.1	16.5	4.0	7.1
4	31.6	4.4	10.0	0.4	4.0
5	33.6	13.4	ND	2.6	ND
6	45.1	6.1	14.6	1.6	7.3
7	38.8	0.1	10.2	3.2	3.2
8	72.3	37.4	55.1	28.3	29.5
9	33.1	2.4	16.5	1.3	3.6
10	ND	16.6	24.1	2.3	5.7
Mean	39.4	11.5	23.0	5.7	7.7

*Percent specific ^{51}Cr release from dengue 2-infected and uninfected cells after 18 hour assay.

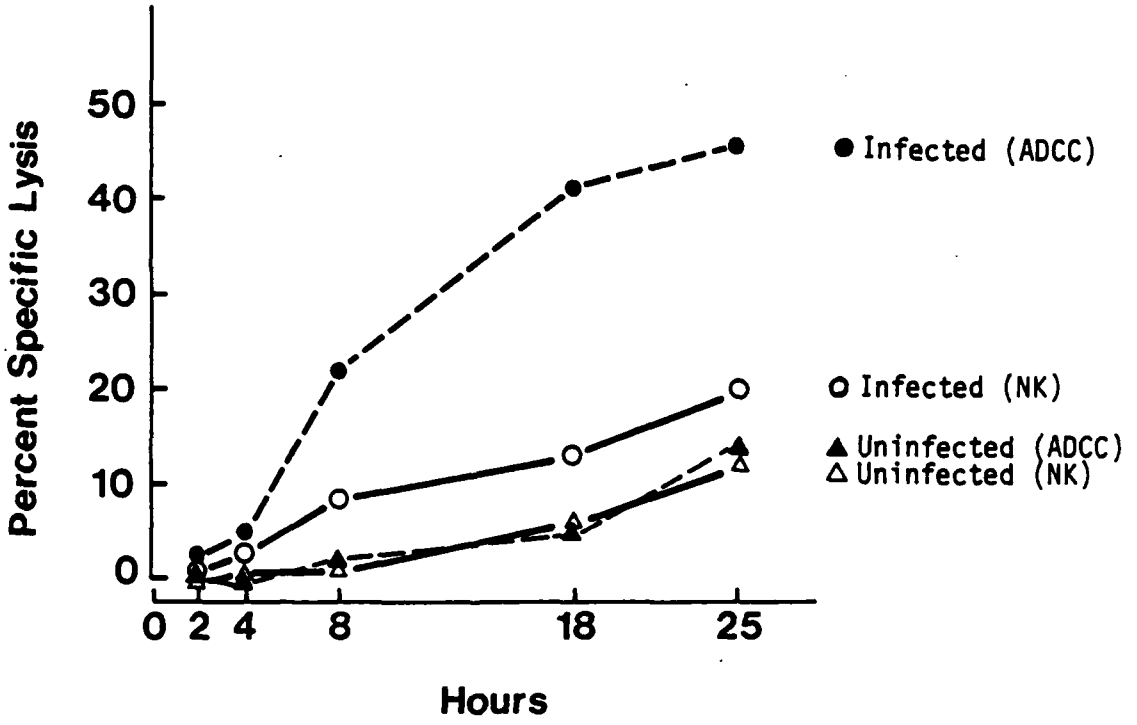
**Ascitic fluid from dengue 2-hyperimmunized mice was used as a source of anti-dengue 2 antibody at 1:20 dilution.

***Significance was determined by paried t test between the lysis of infected Raji cells without antibody and that of uninfected Raji cells without antibody ($p<0.01$). Significance was also determined by paired t test between the lysis of target cells by PBMC with and without antibody ($p<0.0025$ for infected Raji cells, not significant for uninfected Raji cells).

(3) Time course study of the lysis of dengue-infected Raji cells by PBMC and ADCC

Experiments were carried out to determine the time course of lysis of dengue-infected or uninfected Raji cells by PBMC with or without anti-dengue 2 antibody. The specific lysis of PBMC of dengue 2 infected Raji cells reached a maximum at 8 to 18 hours of incubation, depending on the effector cells used. Specific lysis of infected cells by ADCC (percent ^{51}Cr release from infected cells by ADCC minus percent ^{51}Cr release from infected cells by PBMC without antibody) reached a maximum by 18 hours of incubation (Figure 3).

Figure 3. Time course of the lysis of dengue-infected cells by NK and ADCC.



(4) Production of interferon during NK and ADCC assays of dengue-infected Raji cells

We assessed whether interferon was produced during these assays to determine if interferon may have contributed to the enhanced lysis of dengue infected Raji cells by PBMC. High titers of interferon (1600 U/mL) were detected only in the culture supernatants containing both PBMC and dengue-infected Raji cells. The same titer of interferon was also detected in the ADCC assay. However, only 6 U/mL of interferon was detected in the culture supernatant of PBMC and uninfected Raji cells. The interferon was characterized as alpha because it was neutralized by specific antisera to human IFNα, but not by antisera to IFNβ and IFNγ.

Table 2

Quantitation of IFN in culture fluids of effector cells and dengue-infected Raji cells

Target cells	Effector Cells	Anti-Dengue** Antibody	IFN* (U/ml)	% Specific Lysis
Dengue 2-infected Raji	+	+	1600	45.5
	+	-	1600	32.1
	-	-	<6	0
Uninfected Raji	+	-	6	18.8
	-	-	<6	0
None	+	-	<6	-

*Effector cells and target cells were cultured at an E/T radio of 50:1 for 18 hours, and IFN in the culture supernatants was quantitated.

**Hyperimmune mouse ascitic fluid was used at 1:20 dilution.

(5) Addition of anti-IFNα antibody and pretreatment of effector cells with actinomycin D

We examined the effects of anti-IFN antibody on the lysis of dengue-infected Raji cells in the NK assay (Table 3). We added various dilutions of anti-IFNα antibody to the NK assay, and assessed the specific lysis and interferon titer in the culture supernatant at 18 hours. Although the addition of anti-IFNα antibody diluted 1:16 neutralized all detectable IFN produced during the NK assay, the specific lysis of dengue-infected Raji cells did not decrease, suggesting that the presence of interferon throughout the assay was not required to demonstrate the increased lysis of dengue-infected cells.

Table 3

Effect of anti-IFN antibody on the lysis of dengue virus-infected Raji cells by PBMC

Serum added	Dilution	IFN (U/ml)*	% Specific lysis**
None		400 (25)***	36.9 (13.0)***
Anti-IFNα	1:40	<25 (<25)	31.5 (13.0)
	1:160	100 (<25)	36.0 (15.8)
Control	1:40	200 (<25)	35.1 (15.0)
	1:160	400 (50)	31.8 (13.7)

*Dilutions of anti-IFNα antibody were added to wells in the NK assay. IFN in the culture supernatant was quantitated after 18 h of culture.

**E/T ratio was 40:1.

***Numbers in parentheses indicate results obtained by using uninfected Raji cells as target cells.

In order to study the possible early contribution of interferon to the enhanced killing of dengue infected cells, we pretreated the effector cells

with 0.02-0.15 Ug/mL of actinomycin D (Table 4). Although pretreatment of effector cells with actinomycin D decreased the production of IFN by 50 to 75%, the specific lysis of dengue-infected cells did not concomitantly decrease. These results appear to indicate that the enhanced lysis of dengue-infected cells by PBMC may not be due to the interferon produced during the assay.

Table 4

Effect of actinomycin D on the lysis of dengue-infected cells by PBMC and IFN production

ActinomycinD* IFN** (Ug/mL)	% Specific** lysis	(U/mL)
0	31.8	1600
0.02	31.8	800
0.04	31.1	800
0.08	27.7	800
0.16	27.7	400

*Pretreatment of effector cells with actinomycin D is described in Materials and Methods.

**Effector cells and target cells were cultured at an E/T ratio of 50:1 for 18 hours.

II-B Characterization of effector cells

(1) Characterization of effector cells as non-adherent cells

We initially assessed the nature of the effector cells responsible for killing dengue-infected target cells, with or without anti-dengue 2 antibody,

by using adherent cells and non-adherent cells from PBMC (Table 5). The results showed that the predominant cells responsible for the lysis of dengue-infected Raji cells in NK and ADCC assays were non-adherent cells, although some lysis was associated with the adherent cells.

Table 5

Lysis of dengue-infected Raji cells by adherent cells and non-adherent cells with or without anti-dengue 2 antibody

Effector Cells[c] -Ab	E/T Ratio	% Specific ^{51}Cr release[a] Infected Raji +Ab[b]	Infected Raji -Ab	Uninfected Raji -Ab	K562
Unfractionated	50	ND	55.4	43.9	73.7
PBMC	10	47.7	31.7	19.1	33.0
Adherent	50	ND	35.3**	16.7	15.0****
Cells	10	25.5*	16.4***	7.4	10.0*****
Non-adherent	50	ND	51.7**	26.6	75.5****
Cells	10	46.6*	37.0***	18.4	38.8*****

[a]Percent specific ^{51} release from each target after 16 assays.

[b]Hyperimmune mouse ascitic fluid was used at 1:10 dilution.

[c]Percentage of phagocytic cell contained in each fraction is as follows: Unfractionated 15%, Adherent 96%, Non-adherent 3%.

[d]Significance was determined for the difference in the level of lysis between groups with the same symbol.

*$p<0.001$, **$p<0.0025$, ***$p<0.001$, ****$p<0.001$, *****$p<0.001$.

(2) Characterization of effector cells using anti-Leu11 antibody

We first analyzed the effector cells using anti-Leu11 antibody because Leu11 antigen had been reported to be expressed on essentially all functional NK cells in the peripheral blood (3). Treatment of PBL with anti-Leu11b

antibody and complement decreased the level of lysis of dengue virus-infected Raji cells, uninfected Raji cells and K562 cells. The percent decrease in the level of lysis of K562 cells (84% on the average) was significantly greater than that in the level of lysis of dengue virus-infected Raji cells (58% on the average) ($p<0.002$). PBL depleted of Leu11+ cells from most donors did not lyse K562 cells or uninfected Raji cells, but did lyse dengue virus-infected cells to a low but significant level (Table 6, Exp. 1). These results indicate that Leu11+ PBL are the most active effector cell in lysing dengue virus-infected Raji cells, uninfected Raji cells and K562 cells, and that the Leu11- fraction also contains some effector cells which are active in lysing dengue virus-infected Raji cells but which are not active in lysing K562 cells or uninfected Raji cells.

FACS-sorted Leu11+ cells were then used as effector cells. Leu11+ cells were active in lysing dengue virus-infected Raji cells and K562 cells. Leu11- cells did not lyse K562 cells or uninfected Raji cells, but did lyse dengue virus-infected Raji cells to some level (Table 6, Exp. 2). These results are consistent with the results of the complement-mediated cell-depletion experiments.

Table 6

Lysis of dengue virus-infected cells by Leu11$^+$ PBL and Leu11- PBL

Donor	Effector Cells	E/T Ratio	% Specific ^{51}Cr release[a] Dengue Infected Raji	Uninfected Raji	K562
Exp. 1					
D	C'	40	64	11	39
		20	46	8	20
	Anti-Leu11 + C'	40	12***	0***	0***
		20	7***	0***	0***
F	C'	50	41	17	42
		25	27	9	27
	Anti-Leu11 + C'	50	28*	9*	1***
		25	14***	2**	1***
G	C'	30	34	19	54
		15	29	14	30
	Anti-Leu11 + C'	30	23*	9**	8***
		15	12*	7**	5***
H	C'	40	27	15	61
	Anti-Leu11 + C'	40	13*	4***	6***
P	C'	15	21	10	12
		7.5	11	2	8
	Anti-Leu11 + C'	15	7**	1*	1***
		.5	6*	1Σ	1**
Z	C'	40	52	11	39
		20	40	7	17
	Anti-Leu11 + C'	40	17***	2*	2***
		20	12***	1***	0***

(cont.)

Table 6 (cont)

Donor	Effector Cells	E/T Ratio	% Specific ^{51}Cr release[a] Dengue Infected Raji	Uninfected Raji	K562
Exp. 2					
	F Unfractionated	40	32	11	49
		20	21	7	31
	Leu11+	40	ND	ND	ND
		20	16	6	62
	Leu11-	40	10	3	1
		20	8***	2Σ	1***

[a]In experiment 1, PBL were treated with complement (C') alone or anti-Leu11b antibody and C', and then used as effector cells. Significance was determined by Student's t test between the levels of lysis by PBL treated with C' alone and that by PBL treated with anti-Leu11b and C' at the same E/T ratio on the same target cells. In experiment 2, PBM were sorted on a FACS after staining with anti-Leu11b and FITC labelled anti-mouse IgM. Significance was determined by Student's t test between the level of lysis by Leu11+ cells and that by Leu11- cells at the same E/T ratio on the same target cells.

*$p<0.05$, **$p<0.01$, ***$p<0.001$, Σ$p>0.05$ (not significant)

(3) Characterization of the most active Leu11+ cells using anti-Leu7 antibody

We tried to characterize the Leu11+ cells, which contained the most active effector cells, using anti-Leu7 antibody. We found using double staining analysis with the FACS that Leu11+ cells contained Leu7+ cells (Leu11+Leu7+ cells, 3.7% (1.0%-7.8%) of PBL, Leu11+Leu7- cells, 11.1% (2.5%-21.2%) of PBL). FACS-sorted Leu7+ cells and Leu7- cells lysed K562 and dengue virus-infected cells. PBL were also sorted into Leu11+Leu7+ and Leu11+Leu7- fractions with the FACS. Although both Leu11+ Leu7+ cells and Leu11+Leu7- cells lysed dengue virus-infected Raji cells, uninfected Raji cells and K562 cells, Leu11+Leu7-

cells were significantly more active in lysing dengue virus-infected cells than Leu11+Leu7+ cells (Table 7, Exp. 2).

Table 7

Lysis of dengue virus-infected cells by PBL sorted with anti-Leu11 and -Leu7 antibodies

Donor	Effector Cells	E/T Ratio	% Specific ^{51}Cr release¤ Dengue Infected Raji	Uninfected Raji	K562
F.	Unfractionated	20	30	5	29
		10	22	2	21
	Leu7+	20	14	2	29
		10	ND	ND	ND
	Leu7-	20	31**	4*	32Σ
		10	23	3	19
B.	Unfractionated	20	21	9	77
		10	16	6	59
	Leu11+ Leu7+	20	ND	ND	ND
		10	18	8	87
	Leu11+ Leu7-	20	43	19	95
		10	36***	15**	94Σ

¤Significance was determined by Student's t test between the level of lysis of target cells by Leu7+ cells and that by Leu7- cells, and between the level of lysis by Leu11+ Leu7+ cells and that by Leu11+Leu7- cells at the same E/T ratio on the same target cells.

*$p<0.05$, **$p<0.01$, ***$p<0.001$, Σ$p>0.05$ (not significant).

(4) Lysis of dengue virus-infected cells by M1+ PBL

We also characterized the effector cells using OKM1 antibody. M1 antigen has been reported to be expressed on NK cells (4). Treatment of PBL with OKM1 and complement decreased the level of lysis of dengue virus-infected Raji cells, uninfected Raji cells and K562 cells (Table 8, Exp. 1). FACS-sorted M1+ PBL lysed these three target cells, but M1-PBL did not lyse the target cells (Table 8, Exp. 2).

(5) Lysis of dengue virus-infected cells by T3+ cells and T3- cells

The previous experiments using anti-Leu11 antibody gave results which indicated heterogeneity of the cytotoxic lymphocytes, i.e., Leu11+ cells are active in lysing both K562 and dengue virus-infected Raji cells, and Leu11- cells are not active in lysing K562 cells but active in lysing dengue virus-infected Raji cells. These results stimulated us to further characterize the effector lymphocytes in the Leu11-subset.

We demonstrated that Leu11+ cells did not possess a pan T antigen (Leu1) by double staining analysis (data not presented). This result indicates that the Leu11- subset contained all of the T cells which may have contributed to the lysis of dengue virus-infected cells by Leu11- cells. We sorted PBL with the FACS using OKT3 antibody, which also recognizes a pan T antigen, and used them as effector cells (Table 9). T3-cells, which contain Leu11+ cells, lysed dengue virus-infected Raji cells, uninfected Raji cells and K562 cells. T3+ cells did not lyse K562 cells or uninfected Raji cells, however, they lysed dengue virus-infected cells to a low but significant degree ($p<0.02$). These results indicate that T3+ cells contribute to the lysis of dengue virus-infected cells by Leu11- cells.

Table 8

Lysis of dengue virus-infected cells by M1+ PBL

Donor	Effector Cells	E/T Ratio	% Specific ^{51}Cr release[a] Dengue Infected Raji	Uninfected Raji	K562
Exp. 1					
E	C'	35	23	14	45
	OKM1+C'	35	4***	2***	1***
P	C'	40	26	5	41
	OKM1+C'	40	11***	0*	10***
Exp. 2					
A	Unfractionated	10	11	3	4
		5	6	2	3
	M1+	10	21	5	26
		5	12	4	13
	M1-	10	3***	1Σ	1***
		5	2***	1Σ	1***
F	Unfractionated	20	15	5	19
	M+	20	15	9	37
	M-	20	2**	0***	1***

[a] In experiment 1 PBL were treated with complement (C') alone or OKM1 and C', and then used as effector cells. Significance was determined by Student's t test between the level of lysis by PBL treated with C' alone and that by PBL treated with OKM1 and C' at the same E/T ratio on the same target cells. In experiment 2 PBL were sorted on a FACS after staining it OKM1 and FITC-labelled anti-mouse IgG. Significance was determined by Student's t test between the level of lysis by M1+ cells and that by M1- cells at the same E/T ratio on the same target cells.

*$p<0.05$; **$p<0.005$; ***$p<0.001$; Σ$p>0.05$ (not significant)

Table 9

Lysis of dengue virus-infected cells by T3+ cells and T3- cells

Donor	Effector Cells	E/T Ratio	% Specific ^{51}Cr release[a] Dengue Infected Raji	Uninfected Raji	K562
A	Unfractionated	10	20	5	9
		5	15	2	3
	T3+	10	16∞	1	1∞
		5	7∞	1	1∞
	T3-	10	18Σ	5*	33***
		5	14Σ	3Σ	21***
F	Unfractionated	20	20	9	34
		10	16	4	27
	T3+	20	8∞	4	0∞
		10	6∞	1	0∞
	T3-	20	33***	18**	74***
		10	ND	ND	ND

[a]Significance was determined by Student's t test between the level of lysis by T3+ cells and that by T3- cells at the same E/T ratio on the same target cells. *$p<0.05$; **$p<0.005$; ***$p<0.001$; Σ$p>0.05$ (not significant)

∞The level of lysis of dengue-infected Raji cells by T3+ cells was higher than that of K562 cells by T3+ cells determined by Fisher's exact probability test ($p<0.02$).

(6) ADCC activity of Leu11+ cells and T3+ cells

We previously reported that PBL lyse dengue virus-infected cells by ADCC. Anti-dengue antiserum increased the level of lysis of dengue virus-infected cells, but did not increase that of uninfected cells. Normal mouse ascitic fluid did not increase the level of lysis of dengue virus-infected cells or

uninfected cells. These results indicate that the lysis of ADCC is specific for dengue virus-infected cells.

We tried to learn whether Leu11+ cells and T3+ cells, which are two distinct subsets active in lysing dengue virus-infected cells, are also active in ADCC against dengue virus-infected cells. Treatment of PBL with anti Leu11b antibody and complement decreased the level of lysis of ADCC (61% on the average) (Table 10). In experiments using FACS-sorted T3+ and T3- cells as effector cells, T3+ cells were not active in killing dengue virus-infected cells by ADCC, but PBL contained in the T3- fraction were active (Table 11). These results indicate that Leu11+ cells are active in ADCC against dengue virus-infected cells, but T3+ cells are not active.

Table 10

Lysis by ADCC of dengue virus-infected cells by Leu11+ cells

Donor	Effector Cells	E/T Ratio	% Specific ^{51}Cr release[1] + Antibody[2]	-Antibody	X[3]
E	C'	50	29*	19	10
		25	23*	16	7
	Anti-Leu11+C'	50	10*	5	5
		25	7Σ	5	2
F	C'	50	53**	41	12
		25	49***	27	21
	Anti-Leu11-C'	50	32Σ	28	4
		25	28**	14	14'
G	C'	30	53**	34	19
	Anti-Leu11-C'	30	35*	23	12
K	C'	40	25***	17	8
	Anti-Leu11+C'	40	9Σ	7	2
Z	C'	20	51*	40	10
		10	40***	30	10
	Anti-Leu11+C	20	14Σ	12	2
		10	8Σ	5	3

[1]Significance was determined by Student's test between the level of lysis of target cells by PBL with anti-dengue 2 antibody and that by PBL without antibody at the same E/T ratio. *$p<0.05$; **$p<0.005$; ***$p<0.001$; Σ$p>0.05$ (not significant).

[2]Hyperimmune mouse ascitic fluid was used as a source of anti-dengue 2 antibody at a 1:20 dilution.

[3]X means percent specific lysis of target cells by PBL with antibody minus percent specific lysis of target cells by PBL without antibody.

Table 11

Lysis by ADCC of dengue virus-infected cells by PBL contained in T3- fraction, but not in T3+ fraction

Donor	Effector Cells	E/T Ratio	% Specific ^{51}Cr release‡		
			+ Antibody‡‡	- Antibody	X‡‡‡
A	Unfractionated	10	25*	20	5
		5	15Σ	14	1
	T3+	10	14Σ	16	0
		5	8Σ	7	0
	T3-	10	26*	18	
		5	21*	14	/
F	Unfractionated	20	30**	20	10
		10	24*	16	8
	T3+	20	5Σ	8	0
		10	4Σ	6	0
	T3-	20	48*	33	15
		10	ND	ND	ND

‡Significance was determined by Student's t test between the level of lysis of target cells by PBL with anti-dengue 2 antibody and that by PBL without antibody at the same E/T ratio. *$p<0.05$; **$p<0.005$; Σ$p>0.05$ (not signifcant)

‡‡Hyperimmune mouse ascitic fluid was used as a source of anti-dengue 2 antibody at a 1:20 dilution.

‡‡‡X means percent specific lysis of target cells by PBL with antibody minus percent specific lysis of target cells by PBL without antibody.

II-C Comparison of the effector cells with those active in lysing hepatitis A virus-infected cells.

(1) Lysis of hepatitis A virus-infected cells by human PBL

We added human peripheral blood mononuclear cells (PBMC) to hepatitis A-infected and uninfected BS-C-1 cells. PBMC lysed hepatitis A-infected cells to a greater degree than uninfected cells ($p<0.005$), although there was some lysis of uninfected cells in the NK assay (Table 12).

Experiments were carried out to determine whether the effector cells are contained in adherent or in non-adherent fractions, designated as PBL. The results showed that the predominant cells for lysis of hepatitis A-infected BS-C-1 cells were non-adherent PBL (Table 13).

The nature of the PBL responsible for killing the hepatitis A-infected BS-C-1 cells was then analyzed by using anti-Leu11b, OKM1, OKT3, and OKT4 monoclonal antibodies. PBL were reacted with anti-Leu11b, OKT3, or OKT4 antibody and were sorted on the FACS for use as effector cells (Table 14). Leu11+ cells lysed hepatitis A-infected cells and K562 cells; however, Leu11- cells did not lyse either hepatitis A-infected BS-C-1 cells or K562 cells (Expt. 1). T3+ cells and T4+ cells, which did not lyse K562 cells, did not lyse hepatitis A-infected cells. In contrast, T3- cells and T4- cells, which contain Leu11+ cells (data not shown), lysed hepatitis A-infected BS-C-1 cells as well as K562 cells (Expt. 2 and Expt. 3).

We then pretreated PBL with anti-Leu11b or OKM1 antibody and complement, and used them as effector cells (Table 15). Pretreatment of effector cells with anti-Leu11b antibody and complement, and OKM1 and complement, removed their ability to lyse hepatitis A-infected BS-C-1 cells and K562 cells. The results shown in Tables 14 and 15 indicate that the effector cells responsible

Table 12

Lysis of hepatitis A virus-infected BS-C-1 cells by PBMC

Donor	E/T Ratio	% Specific ^{51}Cr release	
		Infected BS-C-1*	Uninfected BS-C-1*
E	40	24.6	29.4
	10	12.5	6.8
F	40	24.8	10.2
	20	9.9	4.4
	10	7.7	1.2
G	120	37.1	29.5
	60	20.5	10.3
I	50	39.8	30.8
	25	30.2	24.0
J	50	32.2	21.9
	25	18.7	10.3
K	25	37.1	27.3

Percent specific ^{51}Cr release was determined after 18 hours incubation. Significance was determined by Wilcoson's rank sum test between the lysis of infected BS-C-1 cells and that of uninfected BS-C-1 cells.

*Statistically significant ($p<0.005$).

Table 13

Lysis of hepatitis A virus-infected BS-C-1 cells by adherent effector cells and non-adherent effector cells

Effector Cells[a]	% Specific ^{51}Cr release		
	Infected BS-C-1	Uninfected BS-C-1	K562
Unfractionated	39.3	30.9	61.7
Adherent	5.8	2.6	8.2
Non-Adherent	40.2*	31.9*	60.5*

[a]Percentage of phagocytic cells contained in each fraction is as follows: unfractionated 18%, adherent 90%, and nonadherent 1%.

[b]Percent specific ^{51}Cr release was determined after 16 hours incubation. E/T ratio was 40. Significance was determined by Student's t test between the level of specific lysis of target cells by the adherent cells and that by the non-adherent cells.

*Statistically significant ($p<0.001$).

Table 14

Lysis of hepatitis A virus-infected BS-C-1 cells by PBL after sorting with anti-Leu11, OKT3 or OKT4 antibody

Effector Cells[a]	% Specific ^{51}Cr release		
	Hepatitis A BS-C-1	Uninfected BS-C-1	K562
Exp 1			
Unfractionated	28.1	12.4	30.5
Leu11+	45.0	22.0	62.4
Leu11-	1.1*	0.5*	0.6*
Exp 2			
Unfractionated	18.7	11.0	29.4
T3+	0.3	0	1.1
T3-	36.4*	34.8*	59.7
Exp 3			
Unfractionated	15.9	9.1	30.7
T4+	0	0	0
T4-	15.7*	10.9*	36.8*

[a]The purity of the cells after sorting was more than 96%.

[b]Percent specific ^{51}Cr release was determined after 16 hours incubation. E/T ratio was 20. Significance was determined by Student's t test between the level of specific lysis of target cells by Leu11+ cells and that by Leu11- cells, between the lysis of T3+ cells and that by T3- cells, and between the lysis by T4+ cells and that by T4-.

*Statistically significant ($p<0.001$).

Table 15

Effect of treatment of PBL with anti-Leu11b or OKM1 antibody and complement on lysis of hepatitis A virus-infected BS-C-1 cells

Treatment of[a] effector cells with C' and antibody to	E/T ratio	% Specific ^{51}Cr release		
		Infected BS-C-1	Uninfected BS-C-1	K562
Exp 1				
--	50	47.4	31.2	41.5
	25	28.1	16.9	26.8
	12.5	15.3	9.2	11.6
Leu11	50	6.2*	1.9*	0.4*
	25	1.3*	0.5*	0.8*
	12.5	1.3*	0.9*	1.1*
Exp 2				
--	40	39.0	27.1	35.5
M1	40	6.2*	6.0*	5.1*

[a]Effector cells pretreated with C' and anti-Leu11b or OKM1 contained less than 1% of Leu11+ or M1+ cells, respectively.

[b]Percent specific ^{51}Cr release was determined after 16 hours incubation. Significance was determined by Student's t test, between the specific lysis of the target cells by PBL treated with C' alone and that by the PBL treated with anti-Leu11 antibody and C' (Exp. 1) and between the lysis by PBL treated with C' alone and that by the PBL with OKM1 and C' (Exp. 2).

*Statistically significant ($p<0.001$).

for lysis of hepatitis A-infected BS-C-1 cells are contained in Leu11+, M1+ and T3- subsets, and that they are contained in the same subsets as the effector lymphocytes that lyse K562 cells.

II-D Serological specificity of ADCC-lysis of dengue virus-infected cells

Dengue 1, 3 and 4-infected Raji cells were lysed by ADCC using homolous anti-dengue antisera as well as dengue 2-infected cells. We used anti-dengue virus type 1, 2, 3, and 4 antisera in ADCC assays. To determine the ADCC titer of each antiserum, sera were diluted and added to ADCC assays. The highest dilution which gave a percent specific ^{51}Cr release greater than that by PBL without anti-serum plus 2 S.D. (standard deviations) was defined as the ADCC titer of the antisera (Figure 4).

Figure 4. Definition of the ADCC titer of the antisera.

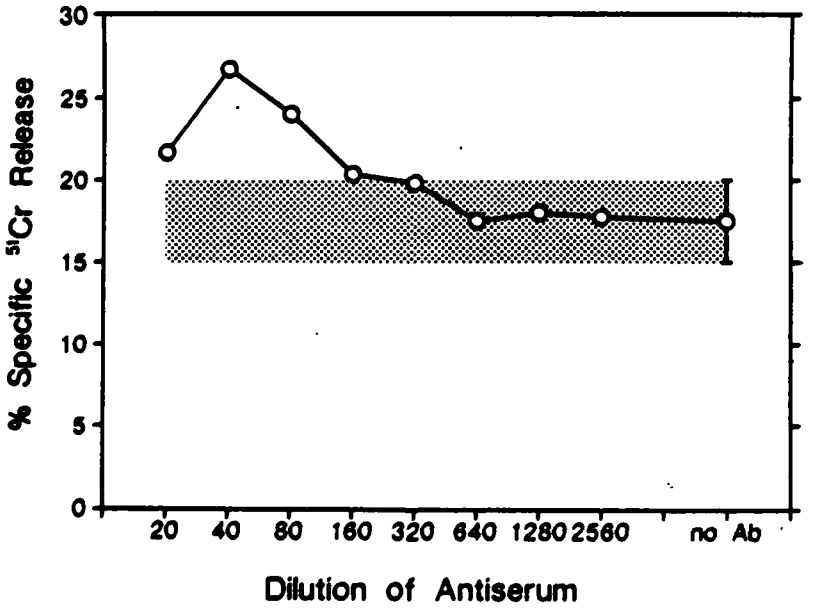


Table 16 shows the FA titer of these antisera. We found using these hyperimmunized murine ascitic fluids that the homologous antiserum was most active in augmenting the lysis of Raji cells infected with virus of the same serotype; however, each antiserum was also active to a lower level in lysing cells infected with other serotypes of dengue virus (Table 17). Therefore, the lysis of dengue virus-infected cells by ADCC appears to be broadly cross-reactive using these polyclonal murine antisera.

Table 16

FA titers of polyclonal anti-dengue antisera detected on dengue virus-infected cell lines

Antisera	FA titer				
	D1-inf	D2-inf	D3-inf	D4-inf	Uninf
Anti-dengue 1	320	40	160	80	<20
-dengue 2	1280	2560	1280	640	<20
-dengue 3	640	320	2560	320	<20
-dengue 4	320	320	320	1280	<20
NMAF	<20	<20	<20	<20	<20

Table 17

ADCC titer of anti-dengue antisera detected on dengue virus-infected cell lines

Antisera	ADCC titer				
	D1-inf	D2-inf	D3-inf	D4-inf	Uninf
Anti-dengue 1	1280	40	160	160	<20
-dengue 2	1280	1280	640	320	<20
-dengue 3	320	40	640	320	<20
-dengue 4	320	320	320	640	<20
NMAF	<20	<20	<20	<20	<20

III. Complement-mediated lysis of dengue virus-infected cells

Antibody-dependent complement-mediated lysis was examined by using dengue virus-infected Raji cells. Polyclonal mouse anti-dengue 2 ascitic fluid was used as antibody. Antibody was absorbed with uninfected Raji cells before use. Human sera from donors who do not have anti-dengue antibodies were used as complement. When dengue-infected cells were cultured with anti-dengue Ab and complement, they were lysed to a low but significant level. Uninfected cells were not lysed by antibody and complement. Neither antibody alone nor complement alone lysed dengue-infected cells or uninfected cells (Table 18).

Table 18

Lysis of dengue virus-infected cells
by anti-dengue antibody and complement

Donor of sera**	% specific ^{51}Cr release*			
	Dengue-infected cells		Uninfected cells	
	+Ab***	No Ab	+Ab	No Ab
Donor 1	10.0	1.3	0.4	0.1
Donor 2	7.2	0	0.8	0.5
Donor 3	6.1	0	0	0

*Percent specific ^{51}Cr release was determined after 1 hour incubation.

**Human sera were used as complement at a 1:16 dilution.

***Hyperimmune mouse ascitic fluid was used as anti-dengue 2 antibody at 1:20 dilution.

Dose response study using serially-diluted antibody showed a good correlation between the percent lysis and the concentration of antibody used (Figure 5). When serially diluted human serum was used as complement with 1:10 diluted antibody, the highest lysis was detected using 1:16 dilution of human

serum (Figure 6). Time course study showed that the specific lysis reached maximum by 2 hours after the beginning of incubation (Figure 7). Although we observed constant lysis of dengue virus-infected cells, the percent specific lysis was around 10 percent at every experiment.

Figure 5. Dose-response relation between antibody-dependent complement-mediated lysis and the dose of antibody.

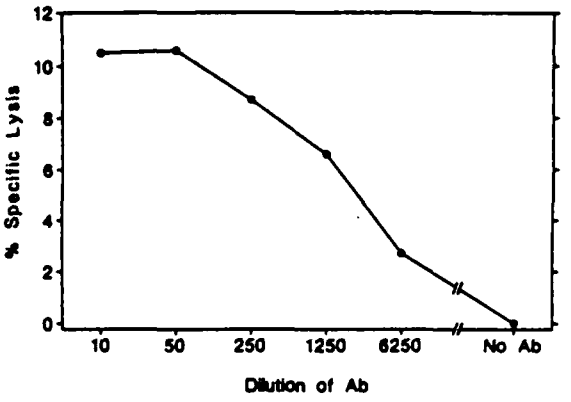


Figure 6. Dose-response relation between antibody-dependent complement-mediated lysis and the dose of complement.

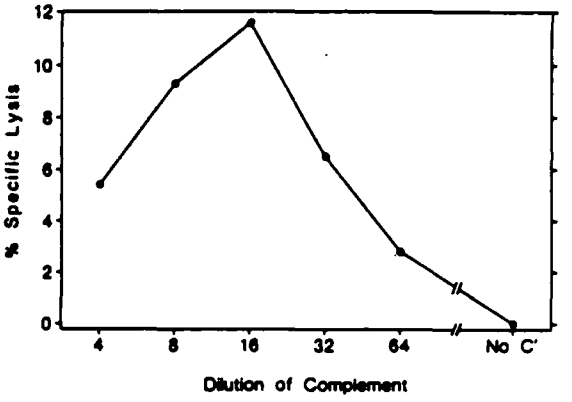
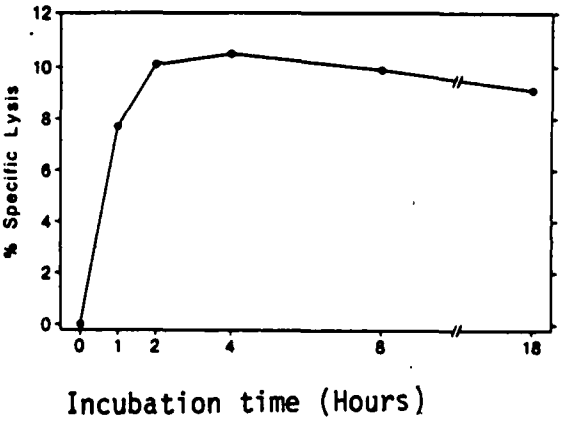


Figure 7. Time course of antibody-dependent complement-mediated lysis.



IV. Interferon induction by dengue virus from human peripheral blood mononuclear cells (PBMC)

IV-A IFN production by dengue virus-infected human monocytes

It has been reported that monocytes are the cells in the peripheral blood which support dengue virus infection (5), and that enhancing antibody increases the number of infected monocytes (6). A current hypothesis suggests that immune-mediated destruction of the dengue virus-infected monocytes leads to the complications of dengue hemorrhagic fever and dengue shock syndrome through the release of chemical mediators from lysed infected monocytes (2).

Monocytes are also known to regular immune responses by producing interleukin 1 and by presenting antigens. Therefore, it is important to elucidate the response of monocytes to dengue virus infection in order to understand human immune responses to dengue virus. We first studied whether monocytes produce IFN when they are infected with dengue virus.

(1) Detection of IFN activity in the supernatants of dengue virus-infected monocytes

Adherent cells are enriched from peripheral blood mononuclear cells of non-immune donors by adherence to plastic petri dishes. These adherent cells were considered as monocytes. The monocytes were infected with dengue virus type 2 at a m.o.i. of 10 per cell in a presence of anti-dengue antiserum diluted $1:2x10^4$, and cultured at 37°C for 1 or 2 days. The culture supernatants were examined for IFN activity. The supernatant fluids of dengue 2 virus-infected monocytes contained IFN at titers from 100 to 600 U/mL. The culture supernatants of uninfected monocytes contained no detectable levels of IFN (Table 19).

Table 19

IFN activity detected in the culture fluids
of dengue virus-infected monocytes

Donor	Dengue-infected monocytes		Uninfected monocytes	
	IFN(U/mL)	%Ag-Positive Cells	IFN(U/mL)	%Ag-Positive Cells
1	400	52	<6	0
2	500	84	<6	0
3	400	69	<6	0
4	100	22	<6	0
5	150	31	<6	0

(2) Time course of IFN-production

We then studied the time course of IFN-production by dengue virus-infected monocytes. IFN activity was detected as early as 12 hours after infection, and reached a maximum on day 2. Dengue antigen was detected as early as 6 hours after infection, and the percentage of antigen positive cells reached a maximum on day 1 (Figure 8).

Figure 8a. Time course of IFN-production by dengue-infected monocytes.

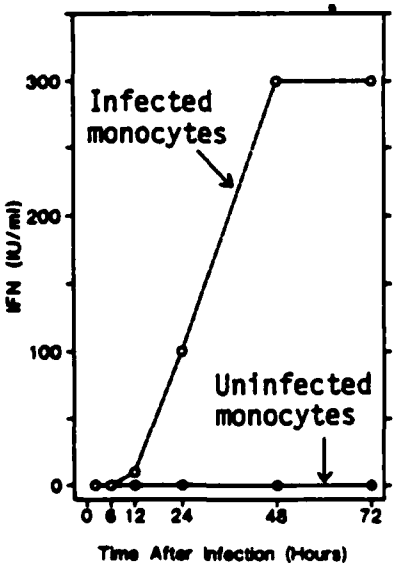
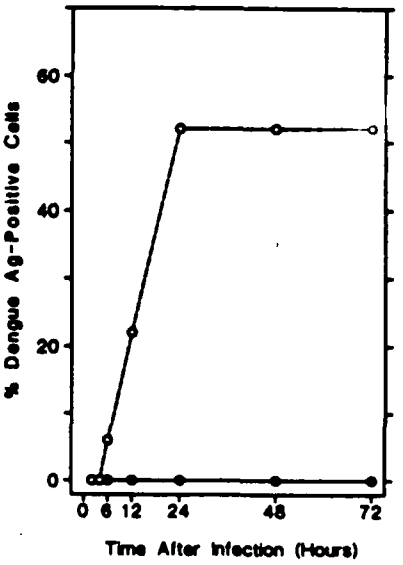


Figure 8b. Time course of the appearance of dengue antigen in infected monocytes.



(3) Characterization of produced IFN

IFN detected in the culture supernatants of dengue virus-infected monocytes were characterized by a neutralization technique using specific antisera to IFNα, IFNβ and IFNγ. IFN was characterized as IFNα because IFN activity was neutralized by antiserum to IFNα, but not neutralized by antisera to IFNβ or IFNγ(Table 20).

Table 20

Neutralization of IFN activity by antisera to IFN

Samples from	IFN (U/ML)			
	Medium	Anti-IFNα	Anti-IFNβ	Anti-IFNγ
Dengue-infected human monocytes	400	<12	400	400

IV-B IFN-induction from non-immune PBL by dengue-infected, autologous monocytes

(1) Detection of IFN activity in the culture supernatants of PBL and dengue-infected, autologous monocytes

We then studied the induction of IFN by dengue-infected monocytes. Monocytes were infected with dengue virus as described above. The infected monocytes were cultured for 2 days, and were used as inducer cells, because the production of IFN by dengue-infected monocytes reached a maximum level on day 2 and they did not produce high titers of IFN after day 2. IFN activity at titers of 200-800 U/mL was detected in the supernatants of culture containing both PBL and dengue-infected monocytes. Very low levels of IFN activity were

detected in the supernatants of PBL cultured alone or dengue virus-infected monocytes cultured alone. When PBL were cultured with uninfected monocytes, only low titers of IFN activity were detected (Figure 9). These results indicate that dengue virus-infected monocytes induce IFN from the PBL of non-immune donors. Pretreatment of the PBL with actinomycin D at a concentration of 0.64 µg/mL decreased the titer of IFN from 800/U/mL to 50 U/mL (Figure 10). This result confirms that the IFN detected in the supernatant fluids was produced by PBL in response to dengue virus-infected autologous monocytes.

Figure 9 Dengue-infected Monocytes induce IFN from non-immune autologous PBL

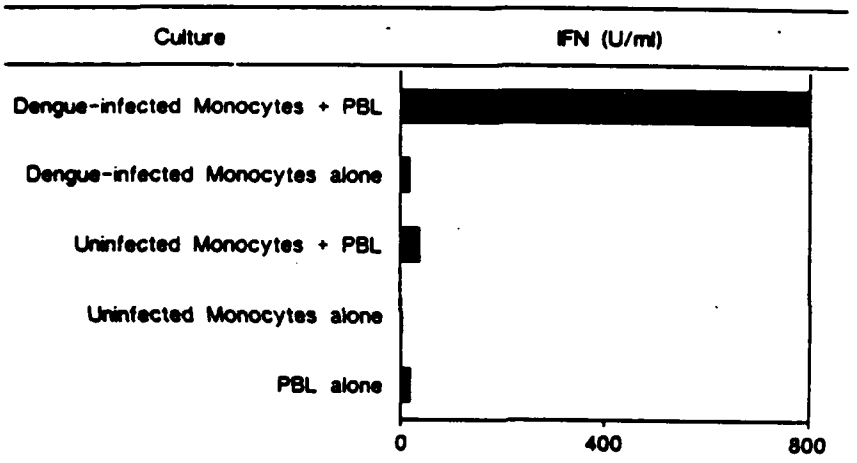
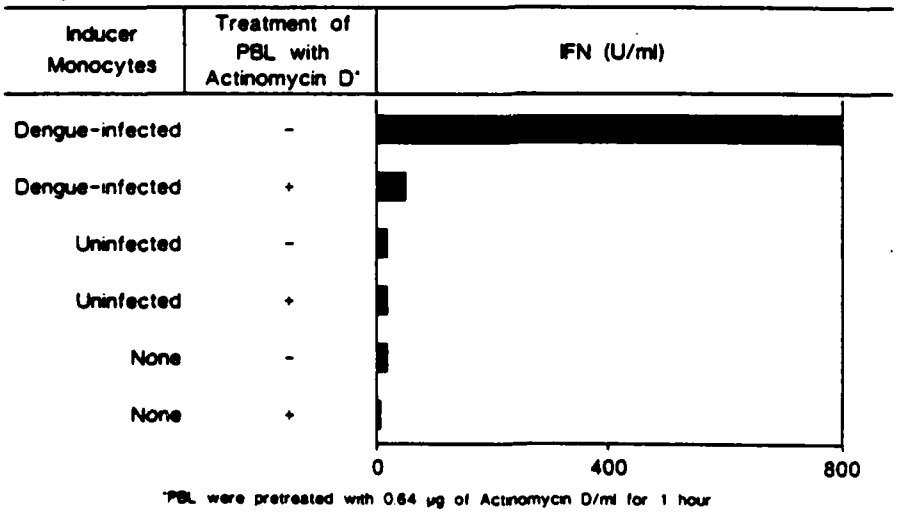


Figure 10 IFN is produced by PBL, not by Monocytes

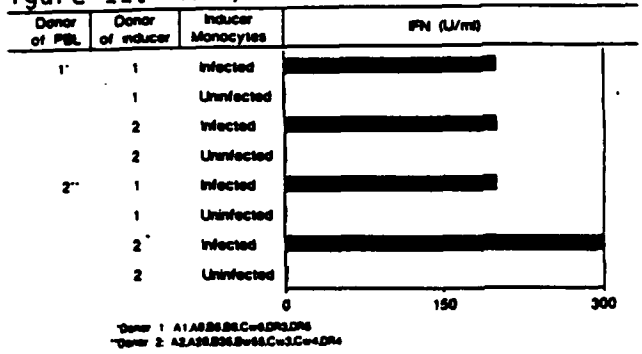


*PBL were pretreated with 0.64 µg of Actinomycin D/ml for 1 hour

(2) MHC-compatibility is not required in the induction of IFN

To determine whether IFN-induction is restricted by MHC-compatibility, we selected two donors who are completely incompatible in their MHC Class I and II antigens. The dengue-infected monocytes of donors 1 and 2 induced similar titers of IFN from PBL of donors 1 and 2. These results indicate that MHC compatibility is not required in IFN-induction by dengue-infected monocytes from non-immune PBL (Figure 11).

Figure 11. IFN induction from PBL by Dengue-infected Monocytes is not MHC-restricted

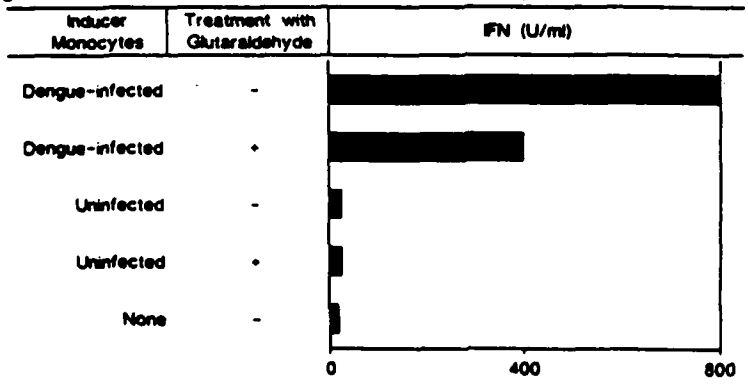


*Donor 1: A1,A68,B8,Cw7,DR3,DR5
**Donor 2: A2,A68,B35,Bw46,Cw3,Cw4,DR4

(3) Glutaraldehyde-treated, dengue-infected monocytes induce IFN from PBL

We examined glutaraldehyde-treated, dengue virus-infected cells for their ability to induce IFN. The glutaraldehyde-treated dengue virus-infected autologous monocytes also induced IFN from PBL. These cells did not produce infectious virus. This result indicates that production of IFN is due to stimulation of PBL by dengue virus-infected cells and is not secondary to infectious dengue virus (Figure 12).

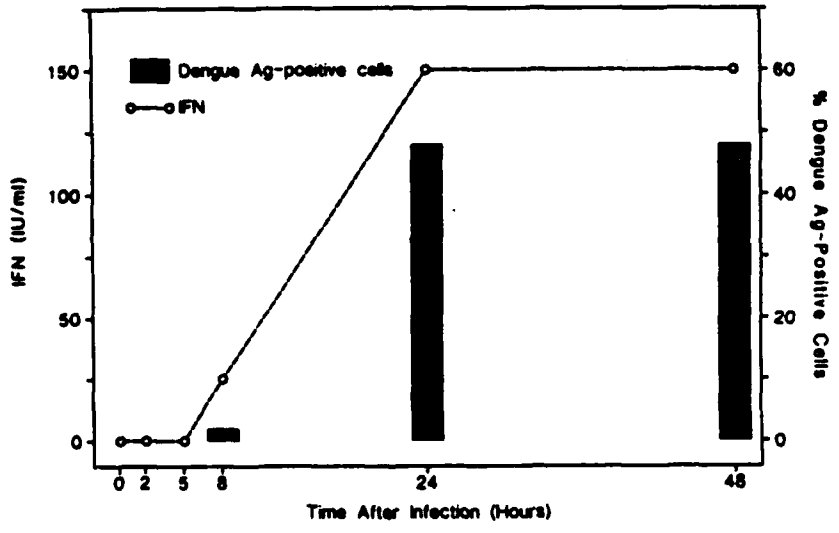
Figure 12. Glutaraldehyde-treated Dengue-infected Monocytes* induce IFN from autologous PBL



*Dengue-infected Monocytes treated with 0.025% glutaraldehyde for 10 min produced no infectious dengue virus

We analyzed dengue virus-infected monocytes at various times after infection to determine the correlation between the ability of IFN-induction and the dengue antigen-expression of infected cells. At intervals after infection, dengue-infected cells were fixed with 3.5% paraformaldehyde before being used as inducer cells. The infected monocytes were able to induce IFN 8 hours after infection when dengue antigens became detectable. The IFN-inducing ability reached a maximum 24 hours after infection when percentage of antigen-positive cells also reached maximum (Figure 13). These results showed good correlation between the antigen-expression and IFN-inducing activity of dengue-infected cells.

Figure 13. Correlation of % dengue antigen-positive cells and IFN inducing ability.



(4) Kinetics and effects of cell concentration of IFN production

The time course study of IFN-production using glutaraldehyde-fixed inducer cells showed that IFN was detected as early as 4 hours after the beginning of incubation, and that IFN titer reached the maximum at 16 hours (Figure 14). The dose-response effect of various number of inducer cells was then evaluated. There was an obvious dose-response relationship between the number of inducer cells and the titer of IFN induced (Figure 15)

Figure 14. Time course of IFN induction from PBL by dengue virus-infected monocytes.

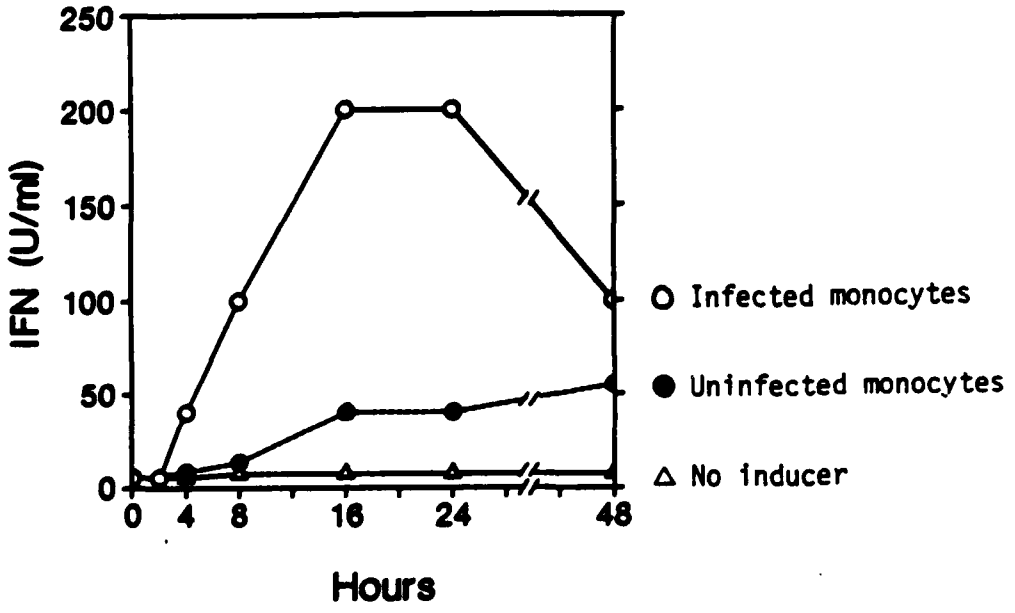
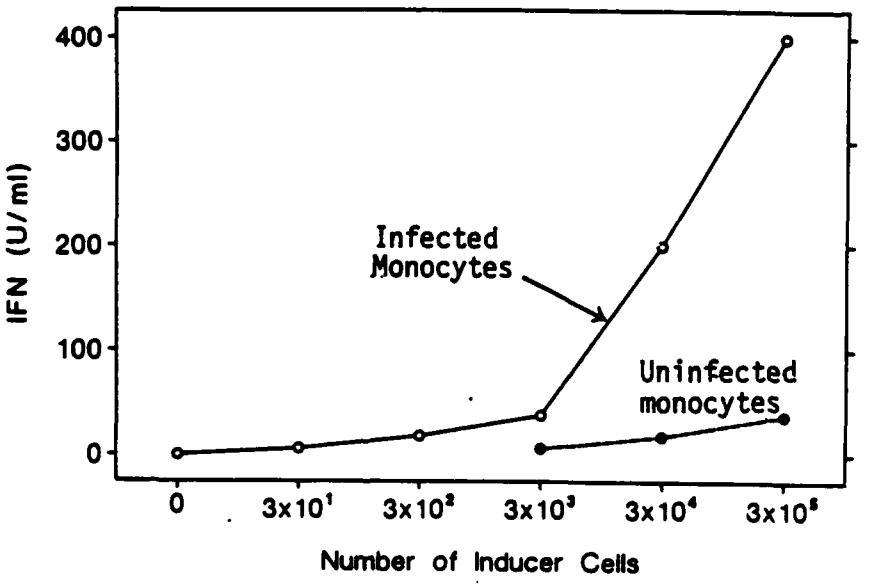


Figure 15. Dose-response relation between the number of dengue-infected monocytes and the titer of IFN induced.



(5) Characterization of produced IFN

The IFNs produced were characterized in a radioimmunoassay using monoclonal antibody to interferons (7). The IFNα was the predominant IFN induced from PBL by dengue-infected monocytes. However, in some experiments low titers of IFN were also detected in the supernatants (Table 21).

Table 21

Characterization by RIA of IFNs induced by dengue virus-infected, autologous monocytes

Culture	IFN (U/ml)		
	RIA		
	IFNα	IFNγ	Bioassay
PBL + Infected Monocytes	250	4	1400
Infected Monocytes alone	<3	<1	<10
PBL + Uninfected Monocytes	<3	<1	<10
Uninfected Monocytes alone	<3	<1	<10
PBL alone	<3	<1	<10

(6) Characterization of IFN-producing PBL using monoclonal antibodies

PBL which produce IFN in response to autologous dengue-infected cells were characterized using 5 monoclonal antibodies; OKT3, anti-Leu11, anti-Leu12, OKM1 and anti-HLA DR antibodies. Table 22 shows the monoclonal antibodies and their specificities.

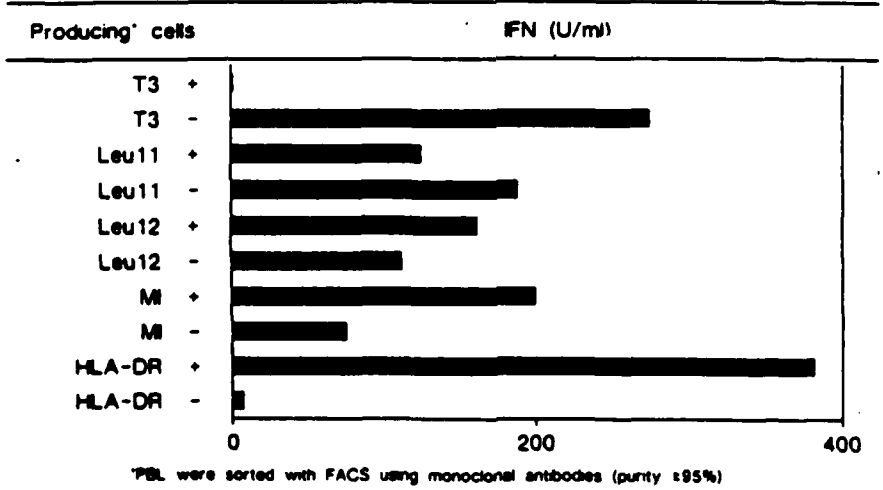
Table 22

Monoclonal antibodies used for characterization of IFN-producing PBL

Monoclonal Antibody	Specificity
OKT3	T cells
Anti-Leu11	NK cells
Anti-Leu12	B cells
OKM1	Monocytes, NK cells
Anti-HLA-DR	Monocytes, B cells, Activated T cells

PBL were sorted by FACS after reduction with these monoclonal antibodies, and were then incubated with inducer cells (Figure 16). HLA-DR+ cells and T3- cells exclusively produced IFN. Neither HLA-DR- cells nor T3+ cells produced IFN. Leu11+ cells and Leu11- cells, Leu12+ cells and Leu12- cells produced almost the same titers of IFN. M1+ cells produced higher titers of IFN than M1- cells. These results indicate that although IFN-producing cells are heterogeneous, they are characterized as HLA-DR+, non T cells.

Figure 16. Heterogeneous PBL produce IFN



*PBL were sorted with FACS using monoclonal antibodies (purity ≥95%)

IV-C IFN-induction from non-immune PBL by dengue virus-infected Raji cells

(1) Induction of IFN by dengue virus infected cells

We then used a dengue-infected B lymphoblastoid cell line (Raji) as the IFN inducer cells. When PBL of non-immune donors were cultured with dengue-virus infected Raji cells, IFN activity was detected at titers from 150 to 800 U/mL in the supernatant fluids. Very low titers of IFN were detected when PBL were cultured alone or with uninfected cells (Table 23). IFN was not detectable in the culture supernatants of dengue virus type 2-infected cells alone or uninfected cells alone.

Table 23

Production of IFN by PBL in response to dengue virus-infected Raji cells

Donor	IFN (U/mL)*		
	Infected Inducer Cells	Uninfected Inducer Cells	No Inducer Cells
A	800	<12	<12
B	400	25	<25
C	800	25	<12
D	200	25	<25
E	600	25	<12
F	150	50	<25

*IFN was assessed by bioassay after 16 hours of incubation. The ratio of PBL:inducer cells was 50:1.

We also examined IFN production using cells infected with dengue virus types 1, 3 and 4 as inducer cells. Cells infected with these other types of

dengue virus induced high titers of IFN similar to those induced by dengue virus type 2-infected cells (Table 24).

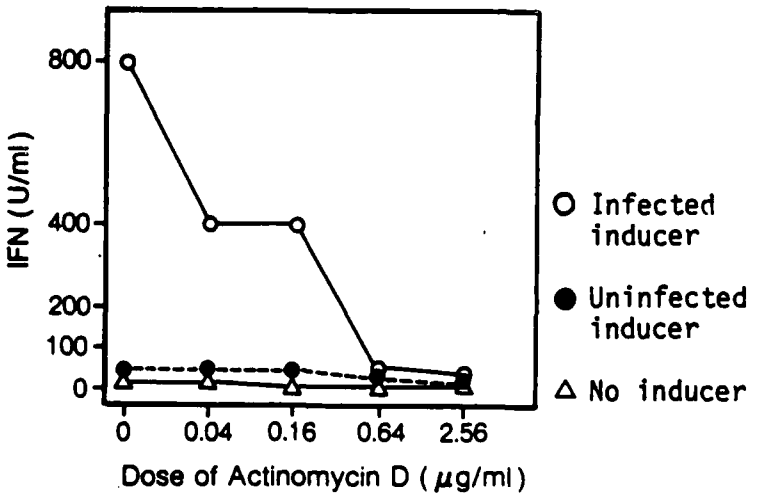
Table 24

Induction of IFN by Raji cells infected with dengue virus types 1, 2, 3 or 4

Donor	Interferon (Units/mL)					
	Dengue 1 Raji	Dengue 2 Raji	Dengue 3 Raji	Dengue 4 Raji	Uninfected Raji	No Inducer
H	300	400	600	1200	<25	<25
L	400	600	800	800	25	<12
P	400	800	800	600	<25	<25
R	600	800	1200	400	12	<12
S	200	800	400	600	25	<25
T	300	150	300	400	50	<25

Pretreatment of PBL with actinomycin D at the doses of 0.64 and 2.56 Ug/mL decreased the production of IFN from 800 to 25 U/mL; however, pretreatment of dengue virus-infected cells did not change the titer of the produced IFN (Figure 17). These results indicate that PBL produce the IFN but not the dengue virus-infected cells.

Figure 17. Abrogation of IFN production by the treatment of PBL with actinomycin D.



(2) Induction of IFN by glutaraldehyde-treated, dengue virus-infected cells

We examined glutaraldehyde-treated, dengue virus-infected cells for their ability to induce IFN. Table 25 shows that the glutaraldehyde-treated cells also induced IFN from PBL. These glutaraldehyde-treated, dengue virus-infected cells did not produce infectious virus during 24 hours of cultivation. In addition, a dengue virus-infected cell line, which produces no infectious virus, also induced a high titer of IFN. These results indicate that production of IFN by PBL is due to stimulation of PBL by dengue virus-infected cells and is not secondary to infectious dengue virus.

Table 25

Induction of IFN by glutaraldehyde-treated, dengue virus-infected cells

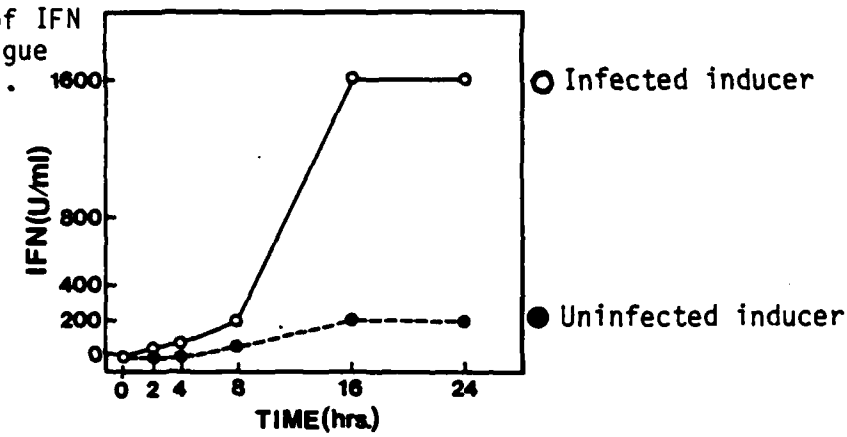
Inducer*	Treatment with Glutaraldehyde	IFN (U/mL) Donor U	Donor R
Infected	-	800	400
Infected	+	300	100
Uninfected	-	38	<25
Uninfected	+	<25	<25
None	-	<25	<25

*PBL were cultured with glutaraldehyde-treated or untreated Raji cells. The ratio of PBL:inducer cells was 40:1. Glutaraldehyde treatment results in one loss of viability of 100% of inducer cells as detected by trypan blue testing. IFN was assessed by bioassay after 16 hours of incubation.

(3) Time course and the effect of inducer cell concentration of IFN production

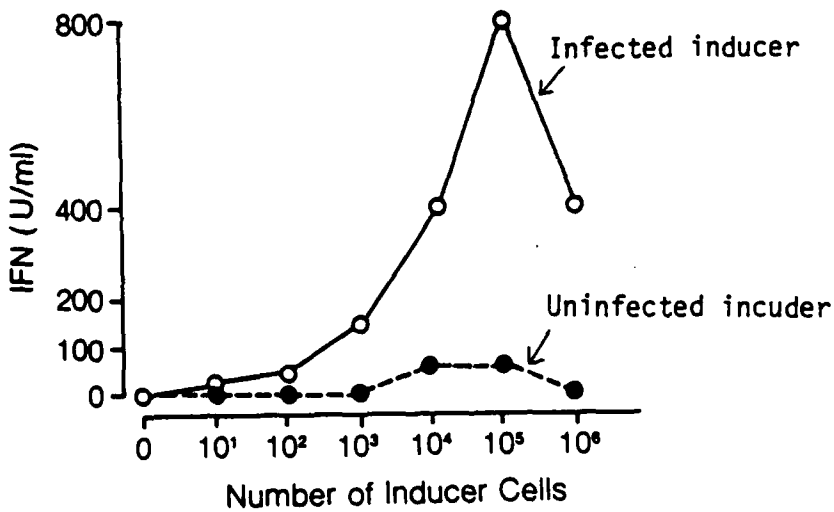
The time course of IFN production was evaluated. IFN was detected as early as 2 hours after the beginning of incubation. The titer reached the maximum at 16 hours of incubation (Figure 18).

Figure 18. Time course of IFN induction from PBL by dengue virus-infected Raji cells.



The dose-response effect of various numbers of inducer cells was then evaluated. 5×10^5 PBL were cultured with 10^1 and 10^6 dengue virus-infected cells for 15 hours (Figure 19). There was an obvious dose-response relationship between the number of inducer cells (from 10^1 to 10^5) and the titer of IFN induced. 10^6 inducer cells are half as effective as 10^5 inducer cells, which may have been due to decreased contact between PBL and inducer cells at such a high concentration of cells.

Figure 19. Dose response relation between the number of dengue-infected Raji cells and the titer of IFN induced.



(4) Characterization of IFN-producing cells using monoclonal antibodies

PBL which produce IFN in response to dengue virus-infected cells were characterized using three monoclonal antibodies; OKM1, anti-Leu11, and OKT3 antibodies. PBL were sorted by FACS after reaction with OKM1, anti-Leu11, or OKT3 antibodies, and were then incubated with inducer cells (Table 26). $M1^+$ and $T3^-$ cells produced higher titer of IFN than did $M1^-$ and $T3^+$ cells, respectively. Leu11+ cells and Leu11- cells produced almost the same titer of IFN. The results indicate that the predominant IFN-producing cells are contained in M1+ and T3- subsets, and that the Leu11+ subset contain some IFN-producing cells.

Table 26

Production of IFN by PBL after sorting with OKM1, anti-Leu11 or OKT3 antibody

Samples from Donor	Effector Cells	IFN (U/mL)* Infected Inducer Cells	Uninfected Inducer Cells	No Inducer Cells
Exp. 1 E	M1+	300	50	<12
	M1-	12	<12	<12
H	M1+	400	50	25
	M1-	50	<6	<6
Exp. 2 H	Leu11+	200	<12	<12
	Leu11-	100	<12	<12
K	Leu11+	100	18	<6
	Leu11-	50	<6	<6
Exp. 3 H	T3+	<25	<12	<12
	T3-	200	<12	<12
K	T3+	12	<3	<3
	T3-	200	<3	<3

*IFN was assessed by bioassay after 126 hours of incubation. Ratio of PBL: inducer cells was 30:1.

(5) Characterization of produced IFN

The IFNs produced were characterized by radioimmuno and bioassays. In the bioassay the produced IFN was characterized as IFNα, because it was neutralized by antiserum to IFNα but was not significantly neutralized by antisera to IFNβ and IFNγ (data not presented). In the radioimmunoassay, however, some IFNγ as well as IFNα was detected in the supernatant fluids obtained from cultures which contained both PBL and dengue virus-infected cells. Although the titer of IFNγ was not as high as that of IFNα, the presence of IFNγ was consistent in all experiments using dengue-infected Raji cells as inducer cells (Table 27).

Table 27

Characterization by radioimmunoassay (RIA) of IFNS induced by dengue virus-infected cells

PBL from Donor	IFN (U/mL)* Infected** Bio Assay	Infected** RIA IFNα$^{\Sigma}$	Infected** RIA IFNγ$^{\Sigma\Sigma}$	Uninfected** Bio Assay	Uninfected** RIA IFNα$^{\Sigma}$	Uninfected** RIA IFNγ$^{\Sigma\Sigma}$	No inducer Bio Assay	No inducer RIA IFNα	No inducer RIA IFNγ
H	1280	208	67	<10	<10	17	<10	<10	9
P	1580	261	30	<10	<10	3	<10	<10	1
R	2000	520	27	<10	<10	7	<10	<10	<1

*IFN was assessed by radioimmuno and bioassays after 16 hours of incubation. Ratio of PBL:inducer cell was 40:1.

**Significance was determined by paired t test between the titres of IFNs induced by dengue-infected inducer cells and those induced by uninfected inducer cells. Statistically significant: $\Sigma p<0.02$, $\Sigma\Sigma p<0.05$.

We then characterized the PBL which produce IFNα and IFNγ using OKM1 and OKT3 antibodies, because these two antibodies most clearly discriminated IFN-producing PBL as shown in Table 26. Treatment of PBL and OKM1 and complement decreased the production of both IFNα and IFNγ as measured by RIA (Table 28). PBL pretreated with OKT3 and complement (T3- cells) produced a higher titer of IFNα and IFNγ than did PBL pretreated with complement alone, probably because of the resulting enrichment of IFN-producing cells. The results indicate that both IFNα and IFNγ are predominantly produced by PBL contained in M1+ and T3- subsets.

Table 28

Characterization of PBL which produce IFNα and IFNγ using OKM1 and OKT3 antibodies

Treatment of Effector	IFN (U/mL) determined by RIA*					
	IFNα			IFNγ		
	Infected Inducer	Uninfected Inducer	No Inducer	Infected Inducer	Uninfected Inducer	No Inducer
Exp. 1 (Donor R)						
C'	270	<10	<10	23	<5	<5
OKM1+C'	100	<10	<10	<5	<5	<5
OMT3+C'	340	<10	<10	<5	<5	<5
Exp. 2 (Donor H)						
C'	330	<10	<10			
OKM1+C'	96	<10	<10			
OKT3+C'	640	<10	<10			
Exp. 3 (Donor P)						
C'				15	5	<1
OKM1+C'				4.5	<1	<1
OKM2+C'				50	7.5	<1

*IFN was assessed by RIA on samples of supernatants removed after 16 hours of incubation. Ratio of PBL:inducer cell was 40:1.

IV-D Inhibition of dengue virus infection by IFN

We showed in the above sections that dengue virus induces IFN from PBMC of non-immune donors by two mechanisms: a) human monocytes produce IFN when they are infected with dengue virus, and b) dengue virus-infected monocytes induce IFN from PBL. The induced IFNs are primarily IFNα. We have begun studies to analyze whether the IFNs produced are actually effective in prevention of dengue virus infection.

Human monocytes and U937 cells were cultured with human IFNα at a concentration of 400 U/mL for 18 hours, and then infected with dengue 2 virus at the m.o.i. of 2 in the presence of anti-dengue 2 antibody. About 40% of the monocytes which were not pretreated with IFN were infected (determined by indirect FA testing) and high titers of IFN, and of dengue virus were detected in the cultures supernatant on day 1 and 2. However, monocytes pretreated with IFNα contained no or a very low percentage of Ag-positive cells and no IFN activity. Yield of the infectious virus was reduced more than 99% below the levels of untreated cultures (Table 29). These results indicate that the levels of IFN which were induced from PBMC by dengue virus can inhibit the further infection of dengue virus to human monocytes.

Table 29

Effect of IFNα on dengue 2 virus infection of human monocytes and U937 cells

Cells Infected	Days After Infection	IFN pretreated		Not pretreated	
		% Dengue Ag Positive Cells	Virus Titer (PFU/mL)	% Dengue Ag Positive Cells	Virus Titer (PFU/mL)
Monocytes	1	0	ND	44	ND
	2	0.8	4.2×10^3	42	3.5×10^6
U937	1	0	ND	15	ND
	2	0.8	4.0×10^3	12	1.2×10^6

V. Discussion

In the first part of this report, we have shown that PBMC-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) effectively kill dengue virus-infected cells in vitro. This conclusion is based on the following results. (a) PBMC from donors without antibodies to dengue 2 virus lysed dengue 2-infected Raji cells significantly better than uninfected Raji cells. (b) Addition of murine anti-dengue 2 antibody into the NK assay increased the lysis of dengue-infected cells, but did not increase the lysis of uninfected cells. Addition of ascitic fluid from unimmunized mice did not increase the lysis of dengue-infected cells. (c) There was a dose-response relationship between the level of anti-dengue 2 antibody added and the specific ^{51}Cr release by ADCC, indicating the dengue virus specificity of the lysis of dengue-infected cells.

We have also detected increased lysis of PBMC of Raji cells infected with another subtype of dengue virus. PBMC lysed Raji cells persistently infected with dengue 4 virus to a higher level than uninfected Raji cells, and addition of anti-dengue 4 antibody further increased the lysis by PBMC of dengue 4-infected Raji cells (unpublished data).

There are reports with several other viruses that infected cells are lysed by NK cells to a greater degree than uninfected target cells (8,9). However, the mechanism of enhanced lysis of virus-infected cells is not clearly known. In some cases, interferon is thought to be a prominent cause of enhanced lysis of infected cells. That is, NK cells which were activated by virus-induced interferon during the NK assay was thought to cause the enhanced filling of infected cells (10). We detected 1600 U/ml of IFN in the culture supernatants of PBMC and dengue-infected cells. However, addition of anti-IFN antibody which neutralized all IFN produced during the assay did not decrease the

specific lysis of infected Raji cells by PBMC. Pretreatment of effector cells with actinomycin D decreased IFN production but did not decrease the lysis of dengue-infected cells. Dengue-infected cells were also lysed to a greater degree than uninfected cells by IFN-pretreated effector cells as when effector cells were not pretreated with IFN. These results suggest that the enhanced lysis of dengue-infected Raji cells by PBMC may not be due to the interferon produced during assay. These results are consistent with reports that natural killing of HSV-1-infected target cells is dissociated from the induction of IFN (11,12). Other possible explanations of the enhanced lysis of virus infected cells include: (1) enhanced adhesion of NK cells to virus-infected targets due to glycoproteins or to virus-induced cellular receptors (13), (b) impairments of infected-cells of repair damaged membranes (14) or (c) an activation of NK cells via viral glycoproteins (15). The mechanism which is responsible for the enhanced lysis of dengue-infected cells by PBMC remains to be elucidated.

We have then characterized the lymphocytes which are active in lysing dengue virus-infected target cells (NK(DV)), in comparison with the lytic activity in the same effector cells for K562 cells. Effector cells contained in Leu11+, M1+ and T3- fractions are the most active in lysing both dengue virus-infected cells and K562 cells. In the Leu11+ subset, Leu11+Leu7- cells are more active than Leu11+Leu7+ cells in lysing target cells. In addition to these effector cells, the T3+ fraction, which does not lyse K562 cells, contains some effector cells which are active in lysing dengue virus-infected Raji cells.

These results indicate the heterogeneity of the PBL which lyse dengue virus-infected cells. Heterogeneity of NK cells has been reported previously using herpes simplex virus-infected target cells. Fitzgerald, et al. reported

that NK cells which lyse HSV-1-infected target cells (HN(HSV)) have somewhat different characteristics from NK cells which lyse K562 cells (NK(K562)) and that NK (HSV) were Leu7+/-, Leu1- and Leu4- (16,17). Hendricks, et al. reported that NK (HSV) were Leu7+ and T3+ (18). Since we may regard Leu1 as T^3, the phenotypes of the predominant NK (DV) are generally consistent with those of the NK (HSV) reported by Fitzgerald, et al. However, we found that T3+ cells were also active in lysing dengue virus-infected cells during highly purified lymphocyte subsets sorted with the FACS. In addition, we observed that the most active NK (DV) are Leu11+ and M1+ cells, and using a double-staining technique we found that Leu11+Leu7- cells are more lytic to dengue virus-infected cells than Leu11+Leu7+ cells.

We described the human PBL active in lysing cells persistently infected with hepatitis A virus. The lysis was due to effector cells in the Leu11+ subset, and T3+ cells did not contribute to the lysis of the hepatis A virus-infected cells. Thus, the lysis was accomplished by a homogeneous subset of Leu11+ cells similar to the subset of the lymphocytes which kill K562 cells, and unlike the heterogeneous subsets of lymphocytes which are responsible for lysis of dengue virus-infected cells.

The relationship between antibody-dependent killer (K) cells and NK cells is also a topic of interest. Most reports indicate that K cells are the same cells as NK cells (19,20), but another report has suggested that K cells are different from NK cells (21) using tumor cells as targets. Our results indicate that most of the PBL which are active in the ADCC against dengue virus-infected cells are contained in the same subsets as PBL active in the NK assay; however, some PBL which are contained in T3+ fraction and active in the NK assay are not active in the ADCC assay.

In the last part of the report, we have described experiments which indicate that dengue virus induces IFN from human PBMC by two mechanisms. Monocytes produced IFN when they were infected with dengue virus. Dengue virus-infected monocytes in turn induce IFN from PBL. Dengue virus-infected monocytes and lymphoblastoid cells (Raji) were also found to induce IFN from PBL of non-immune donors.

The role of IFN produced by PBL exposed to dengue virus-infected cells in dengue infections remains to be studied. It has been reported that mouse serum which contained type 1 IFN activity showed therapeutic effects against dengue virus infection in mice (22). IFNγ has been reported to be more active as an immunoregulatory agent than IFNα and IFNβ. IFNγ but not IFNα or IFNβ induced HLA-DR antigen on human monocytes, and IFNγ was more active in inducing HLA-A and B antigens than IFNα and IFNβ (23). In addition to immunoregulatory effects, IFNγ potentiates the antiviral effect of IFNα and IFNβ (24). It is possible that IFNs may play a role in recovery from dengue virus infection and in the hepatogenesis of DHF and DSS.

The mechanism of IFN-induction by dengue virus-infected cells remains to be elucidated. Glutaraldehyde-treated, dengue-infected cells which do not produce infectious dengue virus induced IFN. In addition, a dengue virus-infected cell line which does not produce detectable infectious dengue virus induced IFN. These results indicate that infectivity of the dengue virus is not essential for the induction of IFN, and it is probable that some component expressed on the infected cells is responsible. Dengue virus has 3 structural proteins; V1, V2 and V3. V3 may be the only exposed protein antigen on the virion and is responsible for hemagglutination (25). It has been reported that the hemagglutinin-neuraminidase glycoprotein of Sendai virus can induce IFN from mouse spleen cells (26). This suggests that dengue viral proteins

expressed in infected cells may be responsible for the induction of IFN. It has also been reported that non-virion proteins are present on the surface of dengue virus-infected cells (27). Recently, it has been reported that monoclonal antibody to the NS1 antigen produced by infection with another Flavivirus (Yellow Fever, strain 17D) fixed complement and lysed infected cells (28). Therefore, it is possible that nonvirion proteins may be responsible for inducing IFNs, but this requires further analysis. Thus, two interesting questions remain to be elucidated. 1) Are proteins expressed on dengue virus-infected cells responsible for the induction of IFN? and 2) What role does the produced IFN play in the immune response to dengue virus? In addition, it is important to define the dengue-specific HLA restricted CTL response to infection, and to determine their role in DHF and DSS. Answers to these questions will lead us to a better understanding of immune responses and their possible role in dengue virus infections.

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